

ENSURING THE MICROBIAL SAFETY OF MINIMALLY PROCESSED FRESH-CUT FRUITS

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ABSTRACT

Strong adherence of bacteria to the cantaloupe surface is favored by irregularities such as roughness, crevices, and pits, thus reducing the ability of washing or sanitizer treatments to remove or inactivate attached cells. The objectives of this study were to compare the efficacy of washing cantaloupes, previously inoculated with *Salmonella* at 4.68 log CFU/cm², with water or 200 ppm chlorine for 2 min with agitation. Washing with water was ineffective in reducing the populations of attached *Salmonella* on cantaloupe surfaces. The efficacy of the chlorine treatment in eliminating *Salmonella* from the cantaloupe surface varied depending on the interval between inoculation and treatment. A 3 log reduction was achieved when treatment was applied at day 0, but at days 3 and 7 of storage at 5 or 25°C, population reductions were approximately 2 logs. From day 3 to 7, the strength of attachment for *Salmonella* on cantaloupe surfaces increased from 0.833 to 0.866 at 5°C and from 0.927 to 0.987 at 25°C. *Salmonella* was not detected in fresh-cut pieces prepared from sanitized melons at day 0, irrespective of day of sanitizer applications to the whole melons, but was detected, mostly in samples sanitized at 7 days post inoculation, when fresh-cut pieces were stored for up to 7 days at 5, 10, 15 or 20°C (Figure 1). In a separate study, apple disks previously inoculated with *Salmonella* were sanitized with solutions of acetic acid, H₂O₂ or trisodium phosphate, individually or in combination, to decontaminate the surface. Only the combination of acetic acid and hydrogen peroxide was effective in reducing the *Salmonella* population on the inoculated apple disks.

INTRODUCTION

For most consumers, cantaloupe melons represent a refreshing and healthy dessert or snack. However, numerous outbreaks of salmonellosis associated epidemiologically with consumption of contaminated melons may lead to reduced sales. Seven melon-related outbreaks involving hundreds of cases have been reported since 1990, and pathogen-contaminated domestic and imported melons have been detected (FDA, 2001a,b;2003). While the largest melon-related outbreaks have been attributed to various *Salmonella* serotypes, other human pathogens including *Escherichia coli* O157:H7, *Campylobacter jejuni* and Norwalk-like virus also have been implicated (Dewaai and Barlow, 2002). *Salmonella* outbreaks in 2000-2002 were traced by the FDA to melons imported from Mexico. On-farm investigations in Mexico conducted by the FDA concluded that "measures were not in place to minimize microbial contamination in

growing, harvesting, packaging, and cooling of cantaloupe” (FDA, 2003). There is an urgent need to develop improved control strategies for eliminating or reducing populations of human pathogens on the surface of minimally processed melons (CDC, 2002).

Several chemical and physical methods have been reported for decontamination of melon before fresh-cut processing, but none eliminates human pathogens attached on the melon surface (Ukuku et al., 2001; Ukuku and Sapers, 2002; Ukuku, 2004). The presence of the raised net tissue of whole cantaloupe gives the surface an inherent roughness that favors microbial attachment and complicates detachment. There is a significant lack of information concerning the ecology of human pathogens on melons. It is not known if human pathogens adhere to melon surfaces via bacterial fimbriae or other bacterial surface components such as exopolysaccharides. Also, the state of human pathogens on the surface of fruits and vegetables may have profound implications as to their susceptibility to antimicrobial treatments. The aim of this study was to investigate the relationship between attachment of *Salmonella* on whole cantaloupe surfaces and efficacy of sanitizer application. Additionally, the efficacy of the sanitizers, applied individually or in combination to apple disks inoculated with *Salmonella*, or *in vitro* to planktonic cells of this pathogen in aqueous suspension, was investigated.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and inoculum preparation. Bacterial strains used in this study were *Salmonella* Stanley H0558, *Salmonella* Newport H1275, *Salmonella* Anatum F4317, *Salmonella* Infantis F4319 (all associated with alfalfa sprout-related outbreaks and obtained from Dr. Patricia Griffin, CDC), *Salmonella* Poona RM2350 (associated with a cantaloupe-related outbreak in 2000 and obtained from Dr. Robert Mandrell, Western Regional Research Center, ARS), *Salmonella* Mbandaka and *Salmonella* Typhimurium (obtained from the USDA-ARS-ERRC culture collection). Bacteria were maintained on Brain Heart Infusion Agar (BHIA, BBL/Difco, Sparks, MD) slants held at 4°C. Prior to use, the cultures were subjected to two successive transfers by loop inocula to 5 ml Brain Heart Infusion Broth (BHIB, BBL/Difco). A final transfer of 0.2 ml was made into 20 ml BHIB with incubation at 36°C for 18 h under static conditions. Bacterial cells were harvested by centrifugation ($10,000 \times g$, 10 min) at 4°C, and the cell pellets were washed in salt-peptone [0.85% NaCl, 0.05 % Bacto-peptone (BBL/Difco)]. A cocktail of *Salmonella* containing $\sim 2.5 \times 10^8$ CFU/ml of each strain was prepared in 3 L of 0.1 % (w/v) peptone-water for each cantaloupe study. The inoculum used for apple disks, containing approximately $\sim 1 \times 10^9$ CFU/ml each of *Salmonella* Mbandaka and *Salmonella* Typhimurium, was prepared as previously described (Liao and Sapers 2000).

Inoculation of cantaloupe/apple. Unwaxed whole cantaloupes (1631g to 1743 g, western shippers) purchased from a local produce warehouse were allowed to come to room temperature ($\sim 20^\circ\text{C}$) overnight before being inoculated. Cantaloupes were submerged in 3 liters of bacterial inoculum ($\sim 18^\circ\text{C}$) and agitated by stirring with a glove-covered hand for 10 min. The inoculated cantaloupes were air dried for 1 h in a biosafety cabinet and then stored at 5 or 25°C for up to 7 days before treatments were applied. Apple disks were prepared from surface-sanitized fruits (Liao et al., 2003) and inoculated with the bacterial inoculum as previously described (Liao and Sapers, 2000).

Attachment study. For the attachment study, 0, 3 and 7 days after inoculation, cantaloupes were washed with water by submerging the melons under the surface of 3 L sterile tap water and then manually rotating the melons to assure complete coverage and contact of surfaces with the wash water for 2 min to remove loosely attached bacteria. Washed melons were placed on

crystallizing dishes inside a biosafety cabinet to dry for 1 h. Bacterial cells in the wash water and those remaining on the melon surfaces were enumerated as described below. The population remaining on the melon surface after washing treatment is described as strongly attached bacteria and is represented by the S_R value, the percentage of total bacterial population strongly attached to the cantaloupe. S_R values were calculated as (strongly attached bacteria)/(loosely + strongly attached bacteria) as reported by (Dickson and Koohmaraie 1999; Ukuku and Fett, 2002).

Sanitizer treatments. To determine the efficacy of chlorine as a sanitizer, 200 ppm chlorine solution was prepared by diluting Clorox® commercial bleach containing 5.25% NaOCl in sterile deionized water and adjusting the pH to 6.4 ± 0.1 by adding citric acid (Mallinckrodt, Paris, KY). Free chlorine in the solution was determined with a chlorine test kit (Hach Co., Ames, IA) that has been approved by the U.S. Environmental Protection Agency. Cantaloupes were dipped in sanitizer solution or water with constant agitation by hand for 2 min. Whole cantaloupes treated at day 0 or 7 days post inoculation were cut into four sections using a sterile knife and the rinds carefully removed. The interior flesh was cut into ~3 cm cubes, and pieces (100 g) were placed in Stomacher® bags (model 400, Dynatech Laboratories, Alexandria, VA) and stored at 5°C, 10°C, 15°C and 20°C.

Inoculated apple disks were immersed in one of five sanitizer solutions: 2.4% acetic acid (AA), 200 ppm NaOCl, 3% H₂O₂, 3% Na₃PO₄, and 3% AA in combination with 3% H₂O₂ for 5 min. Proportions of *Salmonella* cells that were killed (*in vivo* log reduction) following the treatment were determined based on the methods previously described (Liao et al., 2003). For determination of *in vitro* anti-*Salmonella* activities of the five sanitizers, bacterial suspensions in phosphate buffered saline (PBS) containing about an equal number of *S. Mbandaka* and *S. Typhimurium* were exposed to each sanitizer for 5 min. Treated samples were washed twice with phosphate buffered saline (pH 7.1) and then plated onto BHIA to determine the number of cells that survived the treatment, from which the number of cells that were killed and *in vitro* log reduction were calculated.

Sample preparation and enumeration of *Salmonella* from melons. A sterilized stainless steel cork-borer was used to cut rind plugs at random locations on the cantaloupe surface to produce 152 plugs per cantaloupe, each being 22 mm in diameter with a rind surface area (πr^2) of 3.80 cm²; 72 plugs were randomly selected and used for the study. The flesh adhering to the rind plugs was trimmed off using a sterilized stainless steel knife. The plugs were blended (Waring commercial blender, speed level 5, 1 min) with 75 ml of 0.1% peptone-water. Decimal dilutions of the samples were made with 0.1% peptone water, and 0.1 ml was plated in duplicate on XLT4 agar media (BBL/Difco) with incubation at 35°C for 48 h. For fresh-cut cubes, 100 g in 200 ml of PW were homogenized by pummeling in a Stomacher® model 400 (Dynatech Laboratories, Alexandria, VA) for 30 s at medium speed, diluted and plated as above to determine surviving and growth populations of *Salmonella* every two days for up to 10 days at each storage temperature. For comparison, a pure culture of *Salmonella* Poona RM 2350 was plated on XLT4 and run parallel with the samples. Selected black or black-centered colonies from the agar plates were confirmed to be *Salmonella* according to the FDA Bacteriological Analytical Manual following conventional biochemical methods (Andrews et al., 1995) as well as serological assays using latex agglutination (Oxoid, Ogdensburg, New York).

RESULTS AND DISCUSSION

The relationship between the *Salmonella* SR-value and the effectiveness of chlorine treatments is shown in Table 1. Washing with water was not effective in reducing the populations of attached *Salmonella* on cantaloupe surfaces. Chlorine treatment resulted in a 3.4 log reduction at day 0, but by day 3, the efficacy declined, suggesting that the bacterium may have colonized the surface leading to a stronger attachment (Table 1). The strength of attachment for a mixed cocktail of *Salmonella* serovars on cantaloupe surfaces increased from 0.842 to 0.866 during storage for 7 days at 5°C. A similar increase in strength of attachment for *Salmonella* was observed during storage at 25°C, however, the strength of attachment was higher at this temperature. However, the poor correlation between SR-value and log reduction on Day 3 suggests that other factors such as resistance of *Salmonella* in biofilms may be more important than strength of attachment.

Table 1. Strength of *Salmonella* attachment on whole cantaloupe surfaces in relation to days of storage at 5°C and 25°C^a

Storage temperature	SR-values and <i>Salmonella</i> reduction (log CFU/cm ²)		
	0 days post-inoculation	3 days post-inoculation	7 days post-inoculation
5°C	0.842 ± 0.019 (3.42 ± 0.22) ^b	0.833 ± 0.121 (2.55 ± 0.12)	0.866 ± 0.092 (2.28 ± 0.14)
25°C	0.925 ± 0.025 (3.42 ± 0.22)	0.927 ± 0.052 (2.40 ± 0.20)	0.987 ± 0.094 (1.84 ± 0.16)

^a*Salmonella* inoculum was 2.52×10^8 CFU/ml. *Salmonella* recovered from unwashed control cantaloupes were 4.63 log CFU/cm² at day 0, 4.75 log CFU/cm² at day 3 and 4.66 log CFU/cm² at day 7 for all melons stored at 5°C. Populations from cantaloupes stored at 25°C averaged 4.63 log CFU/cm² at day 0, 4.94 log CFU/cm² at day 3 and 4.88 log CFU/cm² at day 7.

^bValues in parentheses represent log reductions after treatment with 200 ppm Cl₂ for 2 min.

Ukuku and Fett (2002) reported similar findings and concluded that bacterial cell surface charge and hydrophobicity appear to be highly correlated to the strength of attachment to the melon surface. The mechanism of attachment of bacterial cells to plant surfaces has been studied most extensively for plant pathogens and symbionts (Romantschuk et al., 1996; Romantschuk, 1992). According to Fletcher (1996) bacterial adhesion occurs in three steps: reversible adsorption, primary adhesion and colonization. During the reversible adsorption phase, the bacterium is at a distance of greater than 50 nm from the substratum and is affected by van der Waal interactions with the substratum. This means that the bacteria can be easily washed off at this stage. At the primary adhesion stage, the distance between the bacteria and the substratum ranges from 10 to 20 nm and the type of force affecting adhesion is electrostatic unless the opposing surface has a net surface charge, then attractive forces will come into play. The colonization step is the final phase for bacterial attachment, and at this point biofilms may be formed which is why killing by chemical means is difficult. According to Buscher et al. (1990), once bacteria overcome the water barrier, and a separation distance of less than 1 nm exists,

additional adhesion interactions such as hydrogen bonding, cation bridging, and receptor-ligand interactions between bacteria and plant surfaces will occur. At this stage the bacteria are difficult to remove. Storage at 25°C was done to simulate contamination of cantaloupe under natural conditions in the field where production of cellulose and curli by *Salmonella* may enable the bacterium to strongly bind to the plant surface and be highly resistant to removal by rinsing or washing steps during processing.

Presence of *Salmonella* was not detected in fresh-cut pieces prepared from sanitized rinds immediately after treatment, irrespective of the day of sanitizer application to the whole melon (Figure 1). However, when fresh-cut pieces were stored at 5, 10, 15 and 20°C for up to 7 days, transferred pathogens were detected, mostly in samples sanitized at 7 days post-inoculation, indicating a reduced ability of chlorine to eliminate the pathogen from the whole cantaloupe surface after storage. This result is consistent with the findings stated above and our previous studies that showed decreased antimicrobial activity of chlorine or hydrogen peroxide in reducing the population of native bacteria and *E. coli* on whole cantaloupe surfaces after storage for 7 days or longer (Ukuku et al., 2001).

Planktonic cells are more susceptible to exposure to antimicrobials than bacterial cells attached on fruit surfaces. The efficacy of five sanitizer solutions in killing *Salmonella* *in vitro* and *in vivo* on apple disks is shown in Table 2. The data in Table 2 showed no direct correlation between the antimicrobial activity of sanitizers against *Salmonella* *in vitro* and *in vivo* attachment to apple disks. A combination of acetic acid and H₂O₂ was the most effective treatment among the five sanitizers tested against attached *Salmonella* on apple disks, approaching a 4-log reduction.

Table 2. Comparison of anti-*Salmonella* activities of five sanitizers *in vitro* and on cut surfaces of apple disks^a

Washed with:	<i>In vitro</i> reduction (log cfu/ml) ^b	<i>In vivo</i> reduction on apple disks (log cfu/disk) ^c
2.4% acetic acid	2.0	1.3
200 ppm sodium hypochlorite	>8.0	1.1
3% hydrogen peroxide	5.3	2.5
3% trisodium phosphate	>7.0	1.5
3% acetic acid + 3% hydrogen peroxide	>8.0	3.7

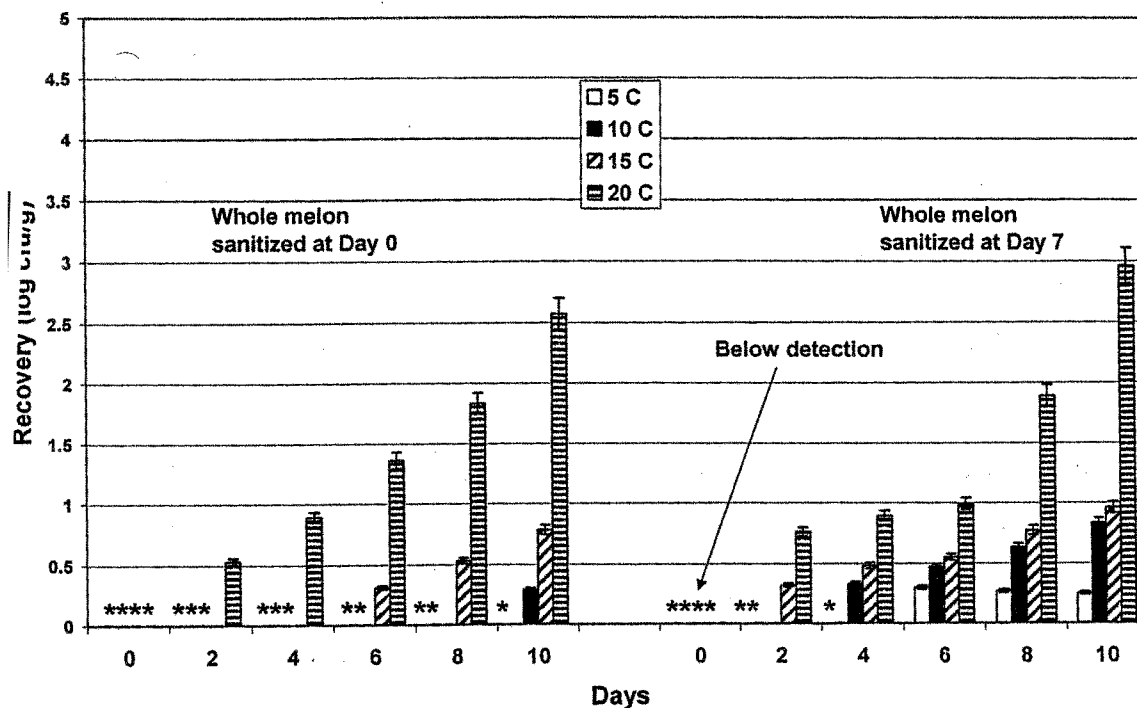
^aThe log reduction values represent the average data of three experiments with two duplicate samples per experiment. Modified from Table 4 in Liao et al. (2003)

^b Cell concentrations in the suspension containing an equal number of *S. Mbandaka* and *S. Typhimurium* were approximately 9.0 log cfu/ml before treatment.

^c Cell numbers on the disk were approximately 7.0 log cfu/disk before the treatment.

In conclusion, once pathogens attach to fruit surfaces, they are much more resistant to sanitizer treatments than planktonic cells. Also, it is important to maintain the cold chain during the fresh-cut preparation of sanitized fruit to inhibit the growth of any surviving pathogens transferred to the fresh-cut pieces, thus reducing the risk of enteric disease.

Figure 1. Population of *Salmonella* in fresh-cut cantaloupe prepared from inoculated cantaloupe sanitized with chlorine (200 ppm) at day 0 or 7 of storage at 25°C



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